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***Brucella* alters the immune response in a *prpA*-dependent manner**

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Abstract

Brucellosis, a disease caused by the gram-negative bacterium *Brucella* sp, is a widespread zoonosis that inflicts important animal and human health problems, especially in developing countries. One of the hallmarks of *Brucella* infection is its capacity to establish a chronic infection, characteristic that depends on a wide repertoire of virulence factors among which are immunomodulatory proteins such as PrpA (encoding the proline racemase protein A or hydroxyproline-2-epimerase), involved in the establishment of the chronic phase of the infectious process that we have previously identified and characterized. We report here that, *in vivo*, *B. abortus prpA* is responsible for an increment in the B-cell number and in the specific antibody response and that these antibodies promote cell infection. We additionally found that *Brucella* alters the cytokine levels of IFN- γ , IL-10, TGF β 1 and TNF α during the acute phase of the infectious process in a *prpA* dependent manner.

1. INTRODUCTION

Many microbial pathogens have the ability to establish chronic infections in their hosts and, as such, must be able to overcome the immune response triggered during the infectious process [1]. Although the manipulation and/or modulation of the immune response by pathogens is currently a well-recognized theme in microbial pathogenesis [2, 3] there still are very few examples of how different pathogens (bacterial, virus or eukaryotic) achieve this task. An accepted hypothesis is that pathogens have evolved sophisticated strategies to subvert the immune response tipping the equilibrium between “response” and “non-response” of the immune system. Many pathogens thus, have achieved a balance consistent with the survival of both the microbe and its infected host by fine-tuning the homeostasis of the latter with no major disturbances [4, 5].

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Brucella spp. are Gram-negative facultative intracellular bacteria that cause brucellosis, a worldwide-distributed zoonosis affecting a broad range of mammals including humans. Brucellosis remains a serious problem in many developing countries, causing important economic losses and human health problems. The infection is characterized by an initial acute phase with flu-like symptoms which, if not treated, can become chronic and persist over the life span of the host causing a broad range of disorders, especially osteoarticular complications [6]. The ability of *Brucella* to establish chronic infections in the face of an ongoing immune response, suggests the existence of bacterial virulence factors with immunomodulatory effects. We have previously described a *Brucella abortus* virulence factor (*prpA*, for Proline Racemase Protein A) that i) is secreted during infection, ii) interacts with NMMII-A in macrophages and iii) induces the release of soluble factors responsible for B-cell proliferation *in vitro* [7, 8]. We also showed that *prpA* is required for the establishment of the chronic phase of infection in mice [8]. This gene has a homologue in *T. cruzi* that also acts as a T-cell independent B lymphocyte mitogen required for virulence [9, 10]. Both genes are hypothesized to act during the acute phase of the infection process, inducing a transient non-responsive state of the immune system that delays or hampers the immune response facilitating chronicity [8, 11]. However, if *prpA* acts as a B-cell proliferator *in vivo*, how it alters host immunity has not been elucidated.

We report here for the first time that *Brucella* infection induces an increment in B-cell number, as has been described during *T. cruzi*'s infection. Moreover, we demonstrate that *prpA* is responsible for this B-cell number increment in infected mice. We also show, *in vivo*, that this virulence factor enhances the production of immunoglobulins directed towards the pathogen and that these antibodies enhance macrophage infection. Finally, we compared the secretion pattern of key inflammatory and anti-inflammatory cytokines in mice during infection with the wild type and the mutant strains and found that they are altered in a *prpA* dependent manner, indicating that this virulence factor also modulates the immune response. Our results show that this gene is clearly involved in the immune modulation process *in vivo* and that alters several aspects of the immune response.

2. MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Escherichia coli strains were grown at 37°C with aeration in LB broth or Terrific broth. *Brucella* strains were grown at 37°C with aeration in Bacto Tryptic soy broth (Becton Dickinson, Sparks, MD). When necessary, media were supplemented with the appropriated antibiotics: ampicillin at 100 µg/ml for *E. coli* and 50 µg/ml for *B. abortus* and gentamicin at 4 µg/ml.

2.2 Infection and inoculation of mice

Infections were carried out as described in [12]. Briefly, female, 60–90 days old BALB/c mice were injected intraperitoneally with 0.2 ml of PBS containing 5×10^4 CFU of *B. abortus* 2308 or *B. abortus-prpA* mutant. For the PrpA-inoculation experiments, BALB/c mice were injected intraperitoneally with 200 µl of PBS or a sterile solution of PrpA (50 µg/ml) in PBS. At different times after infection or inoculation, animals were sacrificed; the

spleens removed, homogenized in RPMI and processed either for direct CFU determination (plating) or fixed and stained for cytometry. All mice were bred in accordance with institutional animal guidelines under specific pathogen-free conditions in the local animal facility (BSL-3, Institute for Research in Biotechnology) of the University of San Martín. Mouse studies were approved by the local regulatory agencies (CICUAE-UNSAM)

2.3 Gentamicin protection assays

J774 A.1 cells were infected as previously described in [13]. Briefly, cells were infected with *Brucella abortus* 2308 with a multiplicity of infection of 20:1 for 1 hr, and Gm and Str (50 and 100 µg/ml) were added to kill non-internalized bacteria. Cells were then washed, lysed with 0,1% Triton X100 and intracellular bacteria were determined by plating dilutions in Difco Tryptic soy agar. For these infections, wild type bacteria were opsonized for 30 min at 37°C with RPMI (control), or sera obtained from 10 days infected mice with either *Brucella abortus* 2308 or *prpA* mutant strains (dilution 1/5000).

2.4 Expression of recombinant PrpA

Recombinant PrpA was produced as previously described [8]. After purification, PrpA was sterilized by filtration through a 0.22µm membrane, and the protein concentration was determined by the Bradford method [14].

2.5 Immunoglobulin quantization

The titer of specific immunoglobulins against *Brucella* was determined by ELISA experiments. Briefly, ELISA Maxisorp plates (Nunc, USA) were sensitized with 0,4 µg/well of a *Brucella abortus* total protein extract overnight and blocked for 2 hrs with 1% BSA in PBS. Serum samples from 10 days post-infected mice were serially diluted, and total immunoglobulins, IgG_{2a} and IgM were detected with HRP-secondary antibodies in a colorimetric reaction and read at 450 nm in a MicroPlate Reader Benchmark (BioRad). The closest absorbance value (450 nm) to 0,5 was multiplied by the dilution factor to obtain the titer. Total immunoglobulin concentration was determined by ELISA (Ebiosciences, USA) according to the manufacturer's protocol.

2.6 Cytokine analysis

Spleens from infected female BALB/c mice were homogenized and frozen at -20°C in 2ml PBS, 1% NP40, 2mM PMSF and 1x protease inhibitor cocktail (Sigma Aldrich), thawed, and centrifuged to remove debris. TNFα, IFNγ, TFGβ and IL-10 concentrations were determined by ELISA according to manufacturer's conditions (eBiosciences).

2.7 Flow cytometry

Spleens from inoculated or infected mice were homogeneized and depleted of red blood cells using Red Blood Cell lysing buffer (Sigma Aldrich). Total splenocytes numbers were quantified using a Neubauer chamber. 10⁶ splenocytes were stained for 30 min. with 1 µl of anti-mouse-CD3e-FITC and 1 µl of anti-mouse-CD19-PE (eBiosciences) for T- and B-lymphocytes respectively. Splenic B- and T-cells were quantified using a CyFlow Space

(Partec). Percentage of B- and T-cells obtained by flow cytometry were multiplied by the total splenocytes number to obtain the total splenic B- and T-cell number.

2.8 Statistical analysis

The differences between the groups were calculated by using the Student's *t* test for normally distributed variables and nonparametric Mann–Whitney test for non-normally distributed variables. *P* < 0.05 was considered statistically significant.

3. RESULTS

3.1 PrpA induces B-cell proliferation *in vivo*

We have previously demonstrated that PrpA acts as a T-cell independent B-cell mitogen when mice splenocytes are treated with the purified recombinant protein *in vitro* [8]. To determine if this proliferation activity also occurs *in vivo*, we inoculated intraperitoneally 50 µg of recombinant PrpA in PBS and measured at 24 hrs post-inoculation the total number of B-lymphocytes in spleens by flow cytometry (see Materials and Methods). As shown in Figure 1A, injection of PrpA significantly increased (doubled) the total B-cell population in 24 hrs indicating that the effect observed *in vitro* can be reproduced *in vivo*. Mitogenicity due to contaminant *E. coli* LPS has been discarded as heat inactivated PrpA or splenocytes from C3H (LPS-non responding) mice do not proliferate (not shown).

To determine if the protein has mitogenic activity in the context of an active infection, we infected mice intraperitoneally with the wild type *B. abortus* and the *prpA* null mutant strains and measured, by flow cytometry at 20 days post-infection, the total number of T- and B-cells in the spleens. Figure 1B shows that the wild type infected mice doubled the B-cell number compared to *prpA* [8] infected or non-infected mice indicating that the gene plays a B-cell mitogenic role during the infectious process. Figure 1C shows the dot plots used to obtain the results shown in panel B.

3.2 PrpA enhances the specific humoral anti-*Brucella* response

Since one of the normal functions of B-cells is the production of antibodies, we evaluated if the *prpA*-induced increment of B-lymphocytes observed during the infectious process also resulted in an increment in total non-specific immunoglobulin titers. We infected mice intraperitoneally with the wild type and the *prpA* mutant and measured in the serum, at 10 days post-infection, the amount of total immunoglobulins and IgG_{2a} by ELISA. As can be observed in Figures 2A and 2B, no differences in the total amount of these immunoglobulins were produced between the animals infected with the wild type and the *prpA* deficient strain. However, when we measured specific antibody titers directed against the pathogen in the same animals, we observed that wild type infected mice (which previously showed increased splenic B-cell numbers) presented significantly higher titers of specific total immunoglobulins and IgG_{2a} compared to the mutant infected animals (Figure 2C and 2D). Altogether these results indicate that *Brucella* alters not only B-cell numbers, but also their function, in a *prpA*-dependent manner.

The results shown above could not be attributed to different bacterial loads between wild type and *prpA* null mutant infected mice during acute infection as both strains showed similar bacterial loads at 1 and 3 weeks post infection (Figure 3A).

3.3 The specific *Brucella* antibody response promotes intracellular replication

The fact that the wild type strain showed higher levels of specific immunoglobulins against *Brucella* in comparison to the *prpA* mutant seemed initially puzzling. Why would a pathogen promote the specific antibody response against itself? Due to the intracellular nature of the infection of *Brucella*, one possible explanation is that these specific antibodies could promote the macrophage uptake. In order to determine if the *prpA*-dependent increase in the antibody titers directed against *Brucella* has an impact in the infectious process, we performed a gentamicin protection assay with J774 A.1 murine macrophage cell line pre-incubating the bacteria 1 hour with serum from uninfected mice or infected with either the wild type or the *prpA* mutant strains. As shown in Figure 3, opsonization of the bacteria with serum from wild type infected mice significantly increased the intracellular bacterial load compared to opsonization with serum from the *prpA* mutant infected or uninfected mice. This result indicates that the increment in the specific antibody titers promoted *in vivo* by PrpA, ultimately promoted the invasion process.

3.4 *PrpA* alters the cytokine pattern during *Brucella* infection

Protective immunity against infection by *B. abortus* is directly related to the induction of a pro-inflammatory or type 1-pattern immune response [15-18]. $\text{IFN}\gamma$ and $\text{TNF}\alpha$ are two central pro-inflammatory cytokines that promote macrophage activation and elimination of intracellular bacteria [16, 19-23]. To determine if the production of these cytokines were altered *in vivo*, mice were intraperitoneally infected with wild type or the *prpA* mutant strains, and the levels of both cytokines were measured in spleens or serum by ELISA. As can be observed in Figure 4, infection with the mutant resulted in increased levels of $\text{IFN}\gamma$ in the serum and in spleens at 7 days post-infection in comparison to the wild type strain (panels A and B). A similar pattern was observed for $\text{TNF}\alpha$, at 7 days post-infection a significant higher concentration of the cytokine was detected in the spleens of mice infected with the mutant strain in comparison with the spleens of mice infected with the wild type strain (Figure 4C). IL-10 and $\text{TGF}\beta 1$ are regulatory cytokines that dampen the protective Type-1 or pro-inflammatory immune response of the host. Therefore, their production is exploited by *Brucella* and many other pathogens to promote infection [24-27].

We infected mice with the *prpA* mutant or the parental strain of *Brucella* and measured IL-10 and $\text{TGF}\beta 1$ levels in their spleens. As can be seen in Figure 4E, spleens from animals infected with the *prpA* mutant strain showed higher levels of IL-10 than the ones from mice infected with the wild type strain at 7 days post-infection. In the case of $\text{TGF}\beta 1$, the *prpA* null mutant showed a statistically lower level of this cytokine in the spleens at 21 days post-infection (Figure 4D). Altogether, these experiments indicate that *prpA* also affects the pattern of pro- and anti-inflammatory cytokines *in vivo*.

5. DISCUSSION

Brucella spp. are pathogens with the capacity to cause chronic infections. This amazing ability to survive in the face of an active immune response highlights the immune modulation capacity of this pathogen.

In this report we have further advanced in the molecular characterization of PrpA as a virulence factor of *B. abortus* (PrpA) that induces a transient anergic state of the immune system and participates in the establishment of a chronic infection [8]. PrpA has hydroxyproline epimerase activity [28], induces T-cell independent B-cell proliferation *in vitro* and is homologous to a B-lymphocyte mitogen of *T. cruzi* that is also involved in virulence [9, 28, 29]. Here we report for the first time that *Brucella* infection produces an increment in the B-cell number and specific immunoglobulin titers and demonstrate that both phenomena are produced *in vivo* in a *prpA* dependent manner. Interestingly, these antibodies enhanced the invasion and intracellular survival of the bacteria in macrophages indicating that the humoral response is actually exploited for the infectious process. It has been clearly established that B-lymphocytes play a role in enhancing *Brucella* virulence, since mice lacking B-cells are more resistant to infection [26]. Additionally, it has also been reported that opsonizing antibodies developed against the pathogen or its LPS also promote infection *in vitro* [30-32], strongly suggesting that the specific humoral response is actually detrimental for the control of the infection.

Cytokines are key effector molecules that orchestrate the immune response of the host. While high levels of anti-inflammatory TGF β 1 and IL-10 have been observed to promote chronic infections [24-27], protective immunity against *B. abortus* is directly related to the induction of pro-inflammatory cytokines [15-18]. Therefore, the strategy of *Brucella* to chronically infect its host seems to be related to its capacity to avoid the establishment of a protective Type-1 response [19, 33-35]. Consistent with this framework, our results show that *prpA* is associated with the down-regulation of INF γ and TNF α and the up-regulation of TGF β 1 *in vivo*, probably skewing the protective pro-inflammatory towards an anti-inflammatory immune response. This may explain the observation that the *prpA* mutant has significantly affected the capacity to establish a chronic infection [8]. In the case of IL-10, it does not seem to support this Type-1-to-Type-2 hypothesis. However, since IL-10 functions to control an excessive and potentially harmful inflammatory response, the higher levels observed in mice infected with *prpA* null mutant could be a physiological consequence of the increased IFN γ observed in these animals [36].

In summary, our data indicate that *Brucella* alters B-cell number and function in a *prpA* dependent fashion. Moreover, we show here that this pathogen exploits B-cell function, specifically antibody production, to its own benefit. Even though infection with the mutant does not show a difference in bacterial load during the acute phase, the fact that the antibodies enhance the macrophagic invasion of the bacteria could indicate that they have a differential distribution in the mouse (i.e. intracellular location) and, thus, are less “visible” to the immune system favoring its persistence. We have also demonstrated that *Brucella* actively alters the cytokine response pattern and that *prpA* is involved in this process indicating that this virulence factor is targeting several arms of the immune response. We

have recently shed some light into the molecular mechanism of PrpA. Although this virulence factor is a B-cell mitogen, we have shown that it actually targets CD11b⁺F4/80⁺ macrophages, where it is translocated during infection [7]. PrpA treated macrophages release soluble factor/s ultimately responsible for B-lymphocyte proliferation. Moreover, we identified NMMHC-IIA as a putative receptor required for PrpA to bind macrophages and exerting its B-cell mitogenic effect. However, the signaling pathways triggered by PrpA in macrophages and the identities of the soluble factors they release still remain elusive. Experiments are in progress to further understand the mechanisms of these pathways for enhancing *Brucella* infectivity.

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Figure 1. *PrpA* induces B-cell proliferation *in vivo*.

A. Determination by flow cytometry of B-lymphocyte number in spleens of mice intraperitoneally inoculated with PBS or 50 μ g of PrpA at 24 hrs post-inoculation. **B.** Determination by flow cytometry of B and T lymphocyte numbers in spleens of uninfected mice or infected with the wild type 2308 strain or the *prpA* null mutant strain at 21 days post-infection. **C.** Flow cytometry dot-plots of B and T cell numbers determined in panel B.

Figure 2. *PrpA* induces specific anti-*Brucella* immunoglobulin production *in vivo*. Determination by ELISA of **A**, total or **B**, IgG2a immunoglobulins in uninfected mice or infected with the wild type 2308 strain or the *prpA* null mutant strain at 10 days post-infection. Determination of **C**, total specific or **D**, IgG2a specific anti-*Brucella* immunoglobulins in uninfected mice or infected with the wild type 2308 strain or the *prpA* null mutant strain at 10 days post-infection.

Figure 3. The specific antibody response promoted by *prpA* enhances bacterial invasion of macrophages.

A. Bacterial load in the spleens of mice infected with the wild type 2308 and the *prpA* null mutant strains during the acute phase of the infectious process does not vary. **B.** Quantification by the gentamicin protection assay of intracellular bacteria 1 hr post-infection with *Brucella abortus* 2308 pre-incubated for 30 min with RPMI or sera from mice infected with either the wild type or the *prpA* null mutant strain at 10 days post-infection. * $P < 0.05$

Figure 4. PrpA alters the levels of pro- and anti-inflammatory cytokines *in vivo*.

ELISA quantitation of IFN γ in serum (A) and IFN γ (B), TNF α (C), IL-10 (D) in spleens of mice infected with the wild type and the *prpA* null mutant strains at 7 days post-infection. Concentration of TGF β 1 (E) in spleens from mice 21 days post-infection.